Ca^{2+}-mediated prostaglandin E_2 induction reduces haematoporphyrin-derivative-induced cytotoxicity of T24 human bladder transitional carcinoma cells *in vitro*

Louis C. PENNING,* Marc J. N. C. KEIRSE,† John VANSTEVENINCK* and Tom M. A. R. DUBBELMAN*‡

*Sylvius Laboratory, Department of Medical Biochemistry, P.O. Box 9503, 2300 RA, and †Department of Obstetrics, Leiden University Hospital, P.O. Box 9600, 2300 RC, Leiden, The Netherlands

The effects of haematoporphyrin-derivative-mediated photodynamic treatment on arachidonic acid metabolism and its relation to clonogenicity have been studied in human bladder-tumour cells. Photodynamic treatment resulted in a transient release of arachidonic acid-derived compounds; prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) especially were strongly increased. This release was reduced by chelation of intracellular Ca^{2+} with Quin-2 or by lowering the extracellular Ca^{2+} concentration in the medium with EGTA, presumably resulting in inhibition of phospholipase A_2. A similar reduction was obtained when indomethacin, an inhibitor of the cyclo-oxygenase pathway, was added prior to light exposure. These three treatments enhanced the photosensitivity, as revealed by the clonogenicity assay. Incubation with PGE_2 prior to light exposure, but not with TXB_2, protected against reproductive-cell death. The results of these experiments suggest that Ca^{2+}-mediated activation of cyclo-oxygenase, resulting in increased levels of PGE_2, participates in a cellular-defence mechanism against photodynamic cell killing.

INTRODUCTION

Photodynamic therapy (PDT) is a new modality for the treatment of malignant tumours. It is based on the light-activation of a photosensitizer, which is retained to higher concentrations in the tumour than in the normal surrounding tissue [1,2]. Experimental evidence indicates that singlet oxygen, generated via type II photochemical reactions, is responsible for most PDT-induced damage [3,4]. Although Photofrin, a purified form of haematoporphyrin derivative (HPD), is now used in phase III clinical trials [5], the biochemical mechanisms leading to photodynamic cell killing are not known. A detailed knowledge of the biochemical background of PDT is important to improve its effectiveness and to reduce side-effects.

Changes in intracellular Ca^{2+} following illumination of sensitizer-loaded cells have been demonstrated [6,7]. It has been reported that the transient increase in intracellular free Ca^{2+} after PDT contributes to enhanced cell survival of aluminium phthalocyanine chloride (AlPc)-sensitized Chinese-hamster ovary (CHO) cells and HPD-sensitized T24 cells [8]. Neither the mechanisms underlying the rise in Ca^{2+} nor its biochemical consequences are known. To investigate cellular responses of the transient Ca^{2+} increase in more detail, HPD-induced photodynamic effects on the Ca^{2+}-dependent arachidonic acid cascade were measured. The questions addressed in the present paper are: does HPD-photosensitization induce a specific set of arachidonic acid metabolites *in vitro*? and what is the function of these metabolites with respect to photodynamic cell killing? A biochemical mechanism that may lead to increased cell survival via an increased cytoplasmic Ca^{2+} concentration and subsequent prostaglandin E_2 (PGE_2) release is described.

MATERIALS AND METHODS

Chemicals

HPD was obtained from Paisley Biochemicals, Paisley, Renfrewshire, Scotland, U.K., and was stored as a stock solution (5 mg/ml) in Dulbecco's phosphate-buffered saline (DPBS) at −20 °C. [5,6,8,9,11,12,14,15(n)-^{3}H]Arachidonic acid (sp. radioactivity 6.7 TBq/mmol) was purchased from New England Nuclear, Den-Bosch, The Netherlands. 2-{[2-Bis(carboxymethyl)-amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline tetrakis(acetoxymethyl) ester (Quin 2-AM) and indomethacin were obtained from Sigma, St. Louis, MO, U.S.A. Reversed-phase guard column and silica-packed analytical columns (Microsphere C_{18}; 3 μm particle size; 100 mm long × 4.6 mm int. diam.) were from Chrompack Nederland, Bergen op Zoom, The Netherlands. Cell-culture products were from Gibco, Breda, The Netherlands, and Greiner, Alphen a/d Rijn, The Netherlands. PGE_2 was obtained from Upjohn, Crawley, Sussex, U.K., and thromboxane B2 (TXB_2) came from ICN/Flow, Zoetermeer, The Netherlands. All other chemicals were of analytical grade and used without further purification.

Cell culture

Human bladder transitional carcinoma cells, clone T24, A.T.C.C. number HTB 4, were kindly provided by Dr. A. H. N. Hopman

Abbreviations used: AlPc, aluminium phthalocyanine chloride; CHO cells, Chinese-hamster ovary cells; DPBS, Dulbecco's phosphate-buffered saline; HPD, haematoporphyrin derivative; 6-oxo-$PGF_{1\alpha}$, 6-oxoprostaglandin $F_{1\alpha}$ [(9α,11α,13*E*,15*S*)-9,11,15-trihydroxy-6-oxoprosta-13-en-1-oic acid]; PDT, photodynamic therapy; PGE_2, prostaglandin E_2 [(5*Z*,11α,13*E*,15*S*)-11,15-dihydroxy-9-oxoprosta-5,13-dien-1-oic acid]; Quin 2-AM, 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline tetrakis(acetoxymethyl) ester; TXB_2, thromboxane B_2 [(5*Z*,9α,11*RS*,13*E*,15*S*)-9,11.15-trihydroxythromboxa-5,13-dien-1-oic acid.

‡ To whom correspondence should be sent.

(University of Maastricht, Maastricht, The Netherlands). Cells were grown attached to Petri dishes in Ham's F10 medium supplemented with 15% (v/v) newborn-calf serum in a humidified CO_2/air (1:19) atmosphere at 37 °C [8].

Cell labelling

Cells were labelled with arachidonic acid as described by De Leo et al. [9]. Briefly, cells were incubated as described above with 1.85 kBq/ml arachidonic acid for 16 h. During light exposure (see below) 1 ml medium samples were collected and analysed for radioactivity with a Packard Tri-Carb 4000 liquid-scintillation counter. After light exposure, DPBS was replaced by culture medium and, at appropriate time points, samples of medium were taken and analysed for radioactivity or used for h.p.l.c. analysis.

Light-exposure

Cells were incubated with 10 μg/ml HPD in DPBS for 1 h at 37 °C prior to illumination. Cells were exposed to light from a 500 W halogen lamp (20 W/m^2). The light was filtered by a cut-off filter ($\lambda > 590$ nm) and a 1 cm circulating water filter (18 °C) to avoid hyperthermic effects.

Cell survival

Prior to light treatment, cells were incubated with 10 μM Quin 2-AM for 1 h. The intracellularly produced Quin-2 leaks out of the cells quite slowly and is certainly still present during illumination [8]. EGTA (0.5 mM) was present in the medium for 6 h after light exposure. Cells were also incubated with 10 ng/ml PGE_2 or with 10 ng/ml TXB_2 for 1 h prior to light exposure. After light treatment cells were trypsin-treated, plated out (20 cells/cm^2) and incubated for about 10 days for colony formation [10]. Colonies were counted after staining with Giemsa reagent. Experiments were done in triplicate and repeated three times. Standard errors were less than 10%.

H.p.l.c.

The experimental set-up consisted of a reversed-phase guard column (75 mm long × 2.1 mm int. diam.) and two silica-packed analytical columns (Microsphere C_{18}). The eluent consisted of 0.75% (v/v) acetic acid in Millipore-filtered water and a gradient of 29–100% acetonitrile. The flow rate was set at 1.0 ml/min. Fractions were collected every minute with a Gilson collector (model 210-B) and mixed with 4 ml of Emulsifier Scintillator 299 from Packard [11].

Histidine oxidation

The oxidation of 2 mM histidine was monitored by measuring the oxygen consumption with a Clark-type oxygen electrode in 50 mM phosphate buffer, pH 7.4, at room temperature.

RESULTS

In the present study the effects of various reagents on photodynamic cell killing were studied. A possible effect of these reagents could be a direct interference with photochemical reactions *per se*. Therefore, these reagents were tested in a model system in which oxidation of 2 mM histidine in 50 mM phosphate buffer, pH 7.4, by 10 μg/ml HPD and light was measured. No effect on HPD-sensitized histidine oxidation was observed.

Figure 1 shows that, during illumination of HPD-loaded T24 cells, arachidonic acid or arachidonic acid-derived metabolites are released into the medium in a light-dose-dependent fashion. Non-sensitized control cells, with or without light exposure, released only a very small amount of radioactivity (up to 100 c.p.m. in 15 min).

As shown in Figure 2, the release of radioactivity by T24 cells, illuminated for 10 min, continued after shutting off the light and replacing DPBS by culture medium. The transient pattern of release was reproducible and occurred following illumination periods varying from 5 to 15 min.

In further experiments the effect of various agents interfering with the arachidonic acid cascade was investigated (Table 1). Chelation of intracellular free Ca^{2+} with 10 μM Quin 2-AM, present during HPD incubation, clearly reduced the release. Also, when after illumination cells were further incubated in culture medium containing 0.5 mM EGTA, release of radioactivity was strongly inhibited. Indomethacin (1 μM), an in-

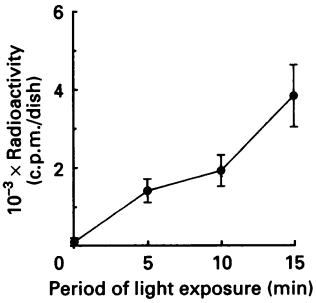

Figure 1 Effect of light exposure times on the release of arachidonic acid and arachidonic acid-derived metabolites from HPD-loaded T24 cells prelabelled with [^{3}H]arachidonic acid

Values for release are means ± S.D. (bars) of triplicate experiments performed in duplicate.

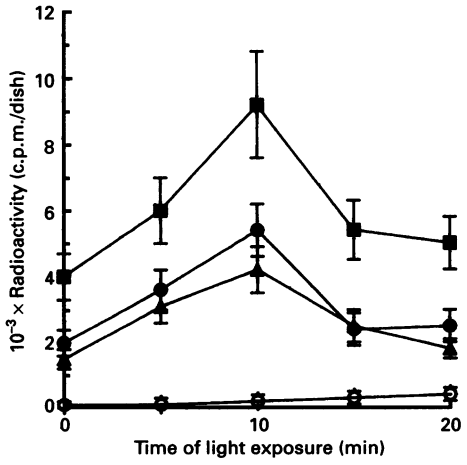

Figure 2 Kinetics of the release of arachidonic acid and arachidonic acid-derived metabolites after HPD photosensitization from T24 cells

Values for release are means ± S.D. (bars) of triplicate experiments performed in duplicate. ○, Dark control; ▲, 5 min light exposure; ●, 10 min light exposure; ■, 15 min light exposure.

Table 1 Effect of Ca^{2+} chelators and a cyclo-oxygenase inhibitor on the release of arachidonic acid or arachidonic acid-derived metabolites from HPD-photosensitized T24 cells

Quin-2AM and indomethacin were added for 1 h prior to light exposure; EGTA was added after light exposure. Release values are means ± S.D. for at least three independent experiments performed in duplicate.

Illumination (min)	Further illumination (min)	Addition	Release [10^{-3} × radioactivity (c.p.m.)]
0	0	None	0.1 ± 0.1
	10	None	0.1 ± 0.1
10	0	None	2.0 ± 0.4
	10	None	5.4 ± 0.9
	10	Quin 2-AM (10 μM)	2.1 ± 0.4
	10	EGTA (0.5 mM)	2.0 ± 0.4
	10	Indomethacin (1 μM)	2.0 ± 0.4

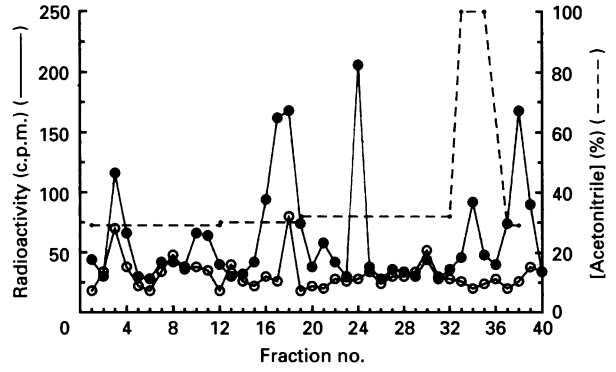

Figure 3 Representative example of a h.p.l.c product profile from HPD-photosensitized T24 cells

○, Samples taken from cells kept in the dark; ●, samples taken from cells illuminated for 10 min and cultured for an additional 10 min. The broken line indicates the acetonitrile gradient. Typical retention times for standards are as follows: 6-oxo-$PGF_{1\alpha}$, 10–11 min; TXB_2, 17–18 min; $PGF_{2\alpha}$ [(5*Z*,9α,11α,13*E*,15*S*)-9,11,15-trihydroxyprosta-5,13-dienoic acid], 21–22 min; PGE_2, 24–25 min; PGD_2 [(5*Z*,9α,13*E*,15*S*)9,15-dihydroxy-11-oxoprosta-5,13-dien-1-oic acid], 28–29 min.

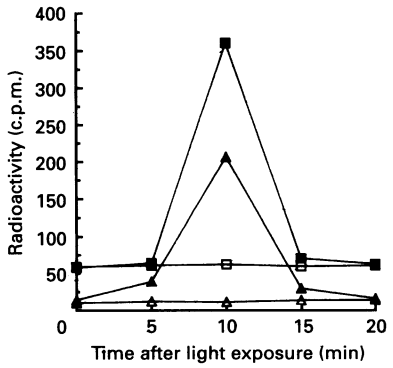

Figure 4 Representative example of the kinetics of PGE_2 and TXB_2 release from HPD-photosensitized T24 cells

Open symbols represent samples from cells kept in the dark; filled symbols represent samples taken from cells illuminated for 10 min and subsequently cultured in the dark. Triangles represent PGE_2; squares represent TXB_2.

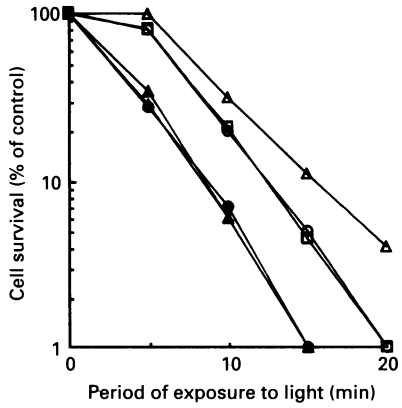

Figure 5 Clonogenicity of photodynamically treated T24 cells

○, No addition; ●, cell incubated with 10 μM Quin 2-AM for 1 h prior to light exposure; ▲, cells incubated with 0.5 mM EGTA in medium for 6 h after light exposure; △, cells incubated with 10 ng/ml PGE_2 for 1 h prior to light exposure; □, cells incubated with 10 ng/ml TXB_2 for 1 h prior to light exposure. Experiments were done in triplicate and repeated three times. S.E.M. values were less than 10%.

hibitor of the cyclo-oxygenase pathway, also significantly reduced the release.

H.p.l.c. analysis of samples of medium yielded elution patterns as shown in Figure 3. For cells kept constantly in the dark, the small amount of radioactivity in the medium was co-eluted with TXB_2. With cells illuminated during 10 min and further incubated in the dark for 10 min (corresponding to the peak in Figure 2) a very strong increase in TXB_2 and PGE_2 and a moderate increase in 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) was observed. Fractions 36–40 contained mainly arachidonic acid and eicosanoids, but were not very well resolved.

The kinetics of TXB_2 and PGE_2 release after photodynamic treatment are presented in Figure 4. The level of TXB_2 increased rapidly about 5–6-fold in 10 min. The increase in PGE_2 was about 10-fold as compared with its concentration immediately after light exposure. After 20 min the concentration of both compounds had decreased to basal levels again. The higher amount of radioactivity in the medium of illuminated cells, 20 min after light exposure, is mainly recovered in the unresolved fractions (36–40).

In Figure 5 the effects of Quin 2-AM, EGTA, indomethacin and externally added PGE_2 and TXB_2 on cell survival of photodynamically treated T24 cells are shown. Apparently photodynamic cell killing was potentiated by 10 μM Quin 2-AM, 0.5 mM EGTA and 1 μM indomethacin, whereas 10 ng/ml PGE_2 had a protecting effect. Externally added TXB_2 (10 ng/ml), on the other hand, did not affect photodynamic cell killing.

DISCUSSION

A better understanding of the biochemical pathways leading to photodynamic cell killing is of the utmost importance to improve PDT. The plasma membrane and mitochondria are sensitive targets for PDT [12–15], but this does not necessarily mean that these are the critical targets in the process of cell killing [16]. Despite almost 20 years of research, no cellular target for PDT-induced cell killing has been established unambiguously. In this context the possible existence of cellular-defence mechanisms against photodynamic cell killing are of interest. The effect of manipulations of such mechanisms on cell survival may help to elucidate the actual cause of photodynamic cell killing. Moreover,

a better understanding of possible defence mechanisms may be interesting from a clinical point of view in the context of developing more efficient PDT protocols.

It was shown previously that photodynamic treatment of several different cell types resulted in a transient increase in cytoplasmic free Ca^{2+} concentration [6,7]. If the increase in intracellular free Ca^{2+} concentration was prevented by intracellular (Quin-2) or extracellular (EGTA) Ca^{2+} chelators, AlPc-sensitized CHO cells and HPD-sensitized T24 cells were rendered more sensitive to photodynamic cell killing [8]. This suggested that the increased intracellular free Ca^{2+} concentrations triggered an as yet unknown cellular-defence mechanism against photodynamic cell killing.

One particular cellular reaction cascade that is known to be activated by increased intracellular Ca^{2+} levels is the arachidonic acid cascade, leading to the synthesis and excretion of several prostaglandins [17]. Increased production of PGs during photodynamic treatment, both of cultured cells and in *in vivo* systems, has indeed been described [18–21]. In *in vivo* systems, these PGs have been implicated in the well-known vascular effects during PDT [19–21].

The present results suggest, however, that the activation of the cyclo-oxygenase pathway may also be involved in cellular defence against photodynamic cell killing. As has been shown, photodynamic treatment of T24 cells led to the release of the arachidonic acid metabolites PGE_2 and TXB_2 into the medium. This release started during illumination (Figure 1) and reached its highest level 10 min after illumination (Figures 2 and 4). Intracellular Quin-2 abrogates the photodynamically induced rise in the intracellular free Ca^{2+} concentration and inhibits the release of arachidonic acid metabolites into the medium (Table 1). Concomitantly it sensitizes T24 cells to photodynamic killing (Figure 5). The same is true for EGTA in the extracellular medium, which also prevents the intracellular Ca^{2+} transient, as shown previously [8]. Inhibition of the cyclo-oxygenase pathway by chelation of Ca^{2+} can be easily explained by suppression of the phospholipase A_2 activity. Incubation with indomethacin, causing an inhibition at the level of cyclo-oxygenase, leads to a similar result; again the production of prostaglandins is inhibited, with a concomitant sensitization of the cells to photodynamic killing (Table 1; Figure 5). Finally, addition of PGE_2 to the medium exhibits a protective effect against photodynamic cell killing. TXB_2, on the other hand, did not protect (Figure 5).

Together these observations strongly suggest the following mechanism. Photodynamic treatment of cells causes a transient increase of the cytoplasmic Ca^{2+} concentration via an as yet unknown mechanism. This Ca^{2+} transient activates phospholipase A_2 and subsequently the cyclo-oxygenase pathway, leading to increased production of prostaglandins, most notably PGE_2 and TXB_2.

Cytoprotective effects of prostaglandins and especially PGE_2 have been described in other cell types, most notably in gastric acid mucosa and hepatocytes [22,23]. PGE_2 is known to increase intracellular cyclic AMP concentrations through its action on adenylyl cyclase, and cyclic AMP has the ability to stabilize plasma membranes [24]. PGE_2 can also maintain cell integrity, irrespective of cyclic AMP, and may render the membrane resistant to changes induced by lipid peroxidation [23]. Such mechanisms may be involved in the cytoprotective effects found here. However, the exact mechanism by which PGE_2 protects against photodynamically induced damage requires further investigation. These results are, as far as we know, the first description of a direct protective effect of PGE_2 on lethal cellular damage induced by photodynamic treatment.

We thank Dr. W. A. Noort for technical assistance on the h.p.l.c. set-up. This work was supported by a grant from the Dutch Cancer Society (IKW 89-01).

REFERENCES

1 Dougherty, T. J. (1987) Photochem. Photobiol. **45**, 4606–4612
2 Spikes, J. D. (1989) in The Science of Photobiology, 2nd edn. (Smith, K., ed.), pp. 79–110, Plenum Press, New York
3 Weishaupt, K., Gomer, C. J. and Dougherty, T. J. (1976) Cancer Res. **36**, 2326–2329
4 Gomer, C. J., Rucker, N., Ferrario, A. and Wong, S. (1989) Radiat. Res. **120**, 1–18
5 Manyak, M. J., Russo, A., Smith, P. D. and Gladstein, E. J. (1988) J. Clin. Oncol. **6**, 380–391
6 Ben-Hur, E., Dubbelman, T. M. A. R. and VanSteveninck, J. (1991) Photochem. Photobiol. **54**, 163–166
7 Specht, K. G. and Rodgers, M. A. J. (1991) Biochim. Biophys. Acta **1070**, 60–68
8 Penning, L. C., Rasch, M. H., Ben-Hur, E., Dubbelman, T. M. A. R., Havelaar, A. C., VanderZee, J. and VanSteveninck, J. (1992) Biochim. Biophys. Acta **1107**, 255–260
9 De Leo, V. A., Hanson, D., Weinstein, I. B. and Harber, L. C. (1985) Photochem. Photobiol. **41**, 51–56
10 Boegheim, J. P. J., Lagerberg, J. W. M., Tijssen, K., Dubbelman, T. M. A. R. and VanSteveninck, J. (1989) Biochim. Biophys. Acta **979**, 215–220
11 Moonen, P., Kolk, G. and Keirse, M. J. N. C. (1983) Prostaglandins **26**, 797–802
12 Santus, R. and Reyftmann, J. P. (1986) Biochimie **68**, 843–848
13 Kessel, D. (1986) Photochem. Photobiol. **44**, 489–493
14 Ceckler, T. L., Bryant, R. G., Penney, D. P., Gibson, S. L. and Hilf, R. (1986) Biochem. Biophys. Res. Commun. **140**, 273–279
15 Atlante, A., Passarella, S., Quagliariello, E., Moreno, G. and Salet, C. (1989) J. Photochem. Photobiol. B: Biol. **4**, 35–46
16 Dubbelman, T. M. A. R. and VanStevenick, J. (1990) J. Photochem. Photobiol. B: Biol. **6**, 345–348
17 Mitchell, M. D. (1988) in Eicosanoids and Reproduction (Hillier, K., ed.), pp. 108–127, MTP Press, Lancaster
18 Henderson, B. Q. and Donovan, J. M. (1989) Cancer Res. **49**, 6896–6900
19 Reed, M. W. R., Wieman, T. J., Doak, K. W., Pietsch, C. G. and Schuscke, D. A. (1989) Photochem. Photobiol. **50**, 419–423
20 Fingar, V. H., Wieman, T. J. and Doak, K. W. (1990) Cancer Res. **50**, 2599–2603
21 Fingar, V. H., Wiemann, T. J. and Doak, K. W. (1991) Int. J. Radiat. Biol. **60**, 303–309
22 Jacobson, E. D. (1992) Gastroenterology **102**, 1788–1800
23 Masaki, N., Ohta, Y., Shirataki, H., Ogata, I., Hayasha, S., Yamada, S., Hirata, K., Nagoshi, S., Mochida, S., Tomiya, T., Ohno, A. Ohta, Y. and Fujiwara, K. (1992) Gastroenterology **102**, 572–576
24 Robert, A. (1976) Adv. Prostaglandins Thromboxane Leukotriene Res. **2**, 507–520

Received 22 October 1992/3 December 1992; accepted 21 December 1992